DEVELOPMENTAL MORPHOLOGY OF ASCOMYCETES

VIII. PETROMYCES ALLIACEUS

by C.V. SUBRAMANIAN and C. RAJENDRAN*

This paper is the eighth in a series on the developmental morphology of the Ascomycetes and deals with *Petromyces alliaceus* Malloch and Cain. The culture used in the present study was received from the late Dr D.I. FENNELL, U.S.-D.A.R.S., USA, under the No NRRL 315. Though this culture is not the type of *Petromyces alliaceus* Malloch and Cain (1972), it is the culture on which the description of the teliomorph of *Aspergillus alliaceus* Thom and Church was given by FENNELL and WARCUP (1959). It is also the culture which was designated as the type of *Syncleistostroma alliaceum* Subram., by SUBRAMANIAN (1972). The present study was well under way before the paper by MALLOCH and CAIN (1972) appeared.

The fungus was grown on Czapek's solution agar medium and the various stages of the development of the teliomorph were studied by making tease mounts stained with 0.1% lactofuchsin as recommended by CARMICHAEL (1955) and sectioning the materials by the paraffin method as described by JOHANSEN (1940) and PURVIS, COLLIER and WALLS (1964). For the study of the anamorph, RIDDELL's (1950) slide culture technique was used and the fungus was stained with 0.5 % solution of Trypan blue in lactophenol (BOOTH, 1971) for 15 minutes. For the study of the germination of ascospores, methods described earlier (SUBRAMANIAN and RAJENDRAN, 1979) were followed.

Memoir No. 259 from the Centre of Advanced Study in Botany, University of Madras.

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DESCRIPTION OF THE FUNGUS

Colonies on Czapek's solution agar at room temperature (23-25°C) attaining a diameter of 6-7 cm in 10 days, consisting of a white basal felt with loosely floccose aerial mycelium.

Hyphae more or less hyaline, thin-walled, branched, remotely to closely septate. Hyphal fusions often noticed. Racquette hyphae present. Stroma ovoid to ellipsoidal or cylindrical, erect, up to 3 mm in vertical axis, 1 mm in diameter, white at first, later becoming grey-black and finally black, white at the tip, composed of thick-walled pseudoparenchymatous tissue which is black on the surface and greenish to white within. Exudate present, collecting in droplets on the stromata. Ascocarps one to several (up to 6 or 7) within each stroma, globose to subglobose or variously shaped, clearly with an opening sometimes, presumably so always, up to 750 x 680μm. Ascocarp peridium around 5μm thick, consisting of a few layers of compactly arranged elongated thin-walled cells. Asci developed from croziers, irregularly disposed, globose to subglobose, 8-spored, evanescent, 12.5-17.0μm diameter (Fig. 5, a). Ascospores hyaline, smooth, globose to subglobose, with a thin equatorial furrow, 5-8 x 4-7μm (Fig. 5, c). Ascospore germination bivalve type. Conidial heads (Fig. 10, y) radiate, splitting into compact divergent columns with age, light yellow at first and later becoming dull golden brown to cinnamon buff. Phialophores simple, variable in size, up to 3 mm long and 15μm broad, smooth, pale yellow, thick-walled, with an apical vesicle. Vesicles globose to subglobose, variable in size, up to 85μm in diameter, bearing metulae and phialides. Metulae mostly 6-12 x 3.0-4.5μm. Phialides borne in groups of up to 8 at the tips of metulae, mostly 8-12 x 1.5-2.0μm. Conidia typically dry, ovoid to subglobose, smooth, yellowish orange, catenate, 2.5-4 x 2-3.5μm (Fig. 10, z).

DEVELOPMENT OF THE TELIOMORPH

The development of the teliomorph is initiated by the production of a very short upright branch arising from an ordinary vegetative hypha on the surface of the medium. This short branch forks, giving rise to a few primary branches which in turn put out rather stiff secondary branches, the whole giving the appearance of a defoliated tree (Fig. 1, a; Plate I, fig. 1). Branching of this kind can be easily spotted in the periphery of young growing cultures when viewed through a stereo-microscope, and can be easily removed with a pair of fine needles, and placed on a microscope slide for further study. Near the centre of this branching system, a few hyphal fusions appear to occur between cells of neighbouring branches (Fig. 1, b). This is followed by profuse branching (Fig. 1, c; Plate I, fig. 2). The resulting branches are short, more or less gnarled and septate. As a result of the profuse branching, an aggregation of hyphae to form a tissue system, the stroma, takes place. The cells of the neighbouring hyphae of the stroma then unite from the centre outwards to form a compact, white mass with a fuzzy complex of protecting hyphae with a tangle of free ends all around (Fig. 1, d; Plate I, fig. 3). This tangle of free ends of hyphae

Fig. 1. – *Petromyces alliaceus*. a-d, stages showing the development of the stroma.

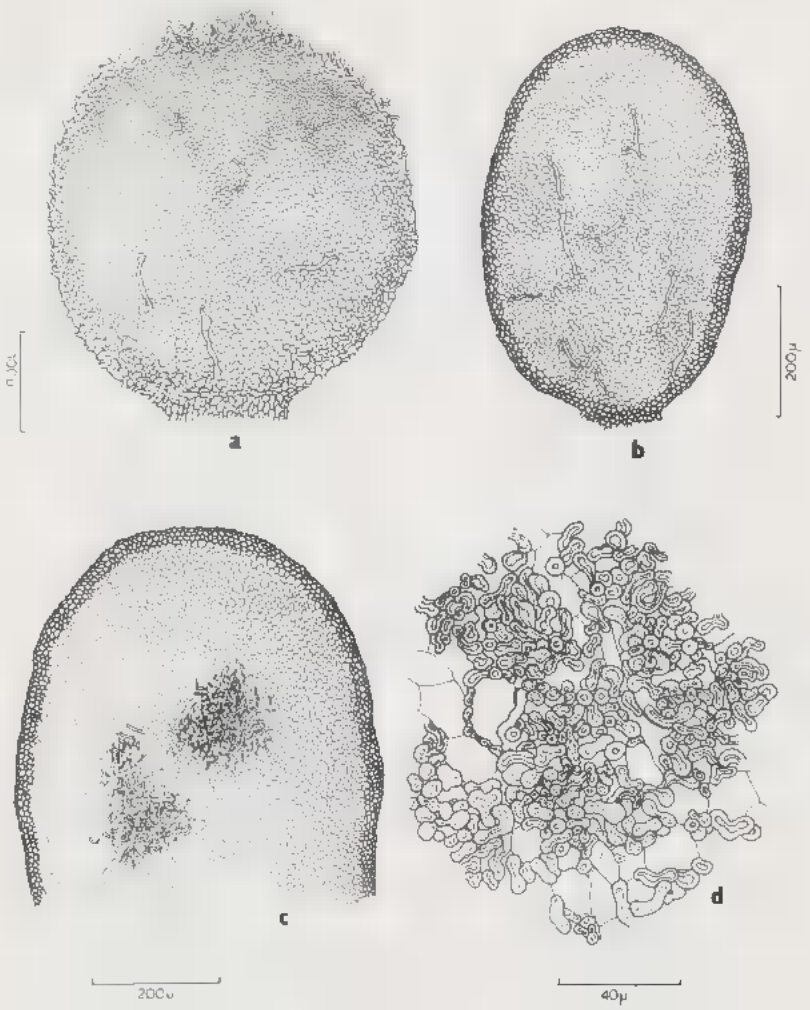

Fig. 2. — *Petromyces alliaceus*. a-b, section of a stroma at a later stage showing its pseudoparenchymatous nature - note the scattered narrow «hyphal bands» - note outer layers of thick walled cells in fig. b; c, part of a stroma showing the development of darkly stained tissue near the «hyphal bands» in the stroma (section); d, enlarged view of the darkly stained tissue within the stroma (section).

is usually shed during later stages of development. The basal hyphal system from which the stroma got differentiated now appear as a tuft of mycelium resembling a stalk and lies embedded in the agar medium.

Sections of a stroma at a later stage of its development shows it to be composed of thin-walled, polyhedral cells with colourless contents compacted together to form a pseudoparenchymatous tissue with a few clearly differentia-

ted, scattered narrow, «hyphal bands» (Fig. 2, a, b), some of which are septate. The remnants of cast-off hyphae which originally formed part of the fuzzy complex of protecting hyphae (Fig. 2, a) in the early development of the stroma are now seen to persist in the periphery of these young stromata. As the stroma matures, the walls of the outermost two or three layers of cells of the stroma become thick, black and evidently hard (Fig. 2, b). During these stages of development, the stroma is still seen attached to the basal tuft of mycelium which now appears compacted and compressed. After a period of such growth and development, the stroma attains a characteristic shape (globose to elongate) and size (up to 750 x 680μm) and there is no further increase in size. At this stage, the stroma is hard, stoney and black and remains without further change for a long period (4-5 months).

The development of the ascocarps within the stroma takes place 4 or 5 months after «maturation» of the stroma. Early in the development of the ascocarps, certain parts of the stroma which are close to the narrow «hyphal bands» become differentiated into darkly stained regions. These darkly stained areas represent groups of irregularly twisted and thick-walled and more deeply stained hyphae (Fig. 2, c; Plate I, fig. 5). Though these hyphae are seen to arise from the neighbourhood of the «hyphal bands», their precise origin could not be traced. These hyphae are frequently septate and branched, though this may not be easily made out in a section; they are seen to lie between cells of the tissues of the stroma, ramifying it conspicuously (Fig. 2, d; Plate I, fig. 6). A section of the stroma at this stage of development shows groups of such distinct darkly stained regions within the pseudoparenchymatous tissue which is surrounded by two or three layers of outer thick-walled, dark cells. Sometimes, these darkly stained hyphae spread in all directions and come to occupy the whole central part of the stroma. When this occurs, the individual darkly stained regions naturally coalesce and so loose their identity as distinct entities.

At this stage, a few cells appear to get lysed and form individual small cavities (Fig. 3, a) within the stroma. These cavities are found usually close to or within the groups of darkly staining hyphae referred to above. Within each of these cavities a differentially staining mass of narrow hyphal threads develop (Fig. 3, b; Plate I, fig. 7) and these form the rudiments of the ascocarp. The exact mode of origin of this hyphal mass is not clear. The stromatic tissue immediately around this darkly staining area shows distortion. This area of distorted cells eventually gets occupied by the growing ascocarp. The peripheral tissue of the ascocarp is now seen to be composed of a closely interwoven mass of distinctly staining hyphae (Fig. 3, c, d). Within the central part of this interwoven mass of hyphae a group of swollen hyphae arise; these are the ascogenous hyphae (Fig. 3, c, f; Plate I, fig. 8). The exact origin of the ascogenous hyphae is not clear. They seem to enlarge from the centre outwards. By a process of septation the ascogenous hyphae give rise to rectangular cells (Fig. 3, g). From each of these rectangular cells a hook-like structure, the crozier (Fig. 4, b, c) develops. Groups of croziers are thus produced. At this stage the ascogenous hyphae and croziers are seen lying in a central cavity within the ascocarp tissue

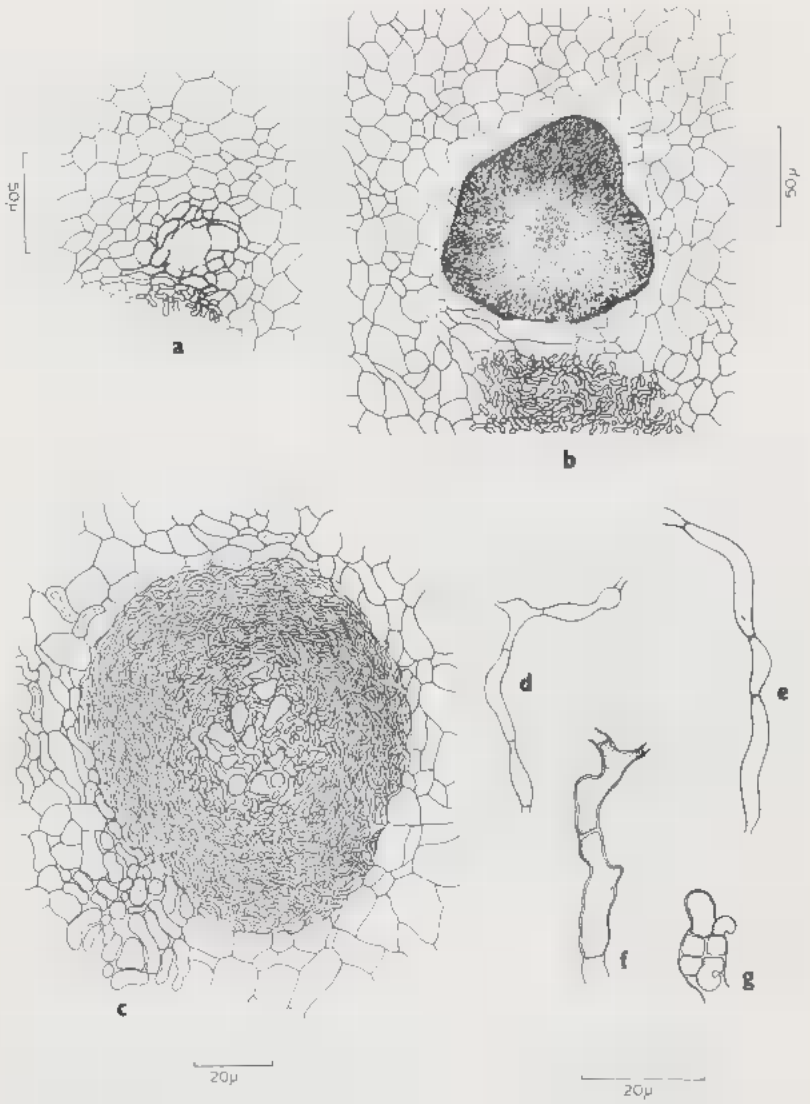

Fig. 3. – *Petromyces alliaceus*. a, early stage in the development of a cavity due to lysis of a few cells in the stroma (section); b, development of the ascocarp rudiments within a cavity in the stroma (section); c, enlarged view of young ascocarp in the stroma showing ascogenous hyphae in the centre surrounded by interwoven narrow hyphae (section); d, e, interwoven narrow hyphae (teased out); f, ascogenous hypha; g, crozier development.

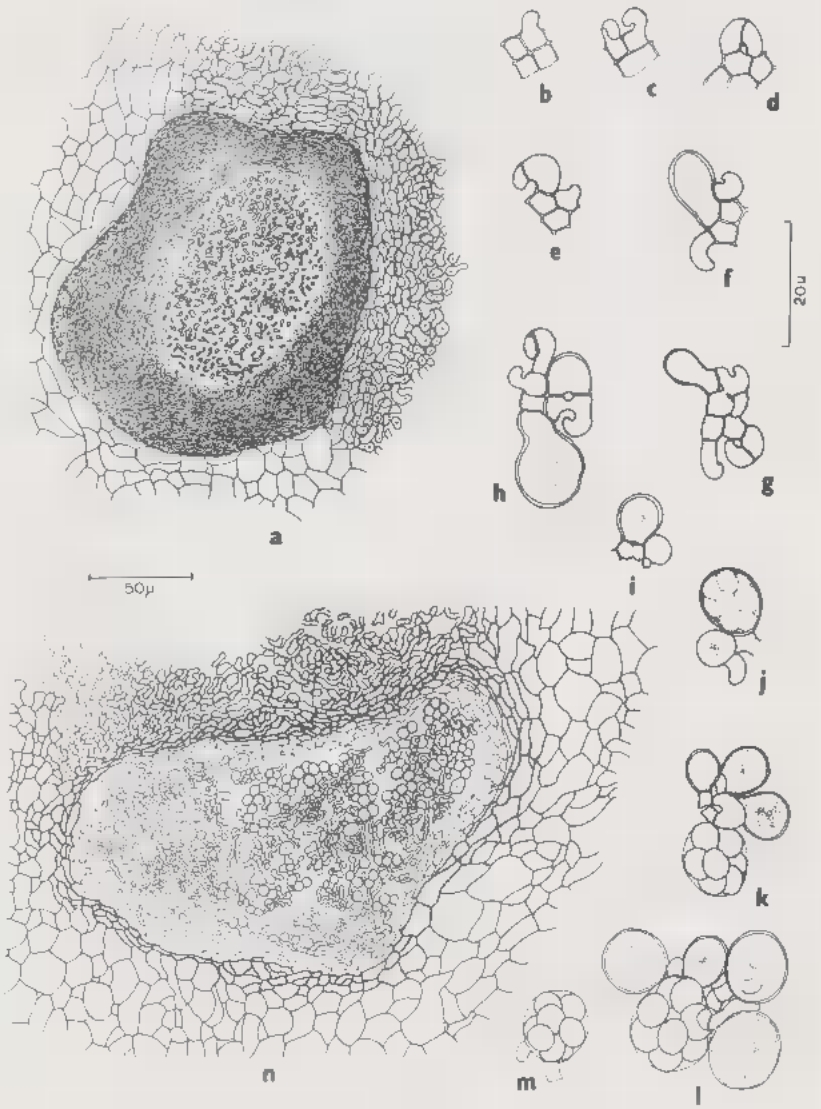

Fig. 4. – *Petromyces alliaceus*. a, section of an ascocarp in ■ stroma showing enlargement of the central cavity with ascogenous hyphae and croziers within; b-k, development of the croziers and asci - note fusion of basal and terminal cells of croziers in fig. g, h; l, a group of asci; m, a mature ascus; n, ■ mature ascocarp with a hyaline peridium within the stroma (section).

(Fig. 4, a; Plate I, fig. 9). Concurrent with the further development of ascogenous hyphae and crozier formation within the ascocarp, the size of the central cavity of the ascocarp naturally increases; in consequence, there is also a gradual reduction and thinning of the surrounding hyphal system.

Formation of two septa in each crozier now divides it into three cells, the basal, the penultimate and the tip cells (Fig. 4, d, e). The penultimate cell of the crozier develops into an ascus (Fig. 4, f); in most cases, the apical and the basal cells fuse and form another crozier (Fig. 4, g, h), which later becomes 2-septate and, as usual, the penultimate cell of this crozier also develops into an ascus and remains close to the first one (Fig. 4, i, j). This process may be repeated several times so that a cluster of asci is formed (Fig.4, k, l); these asci are arranged irregularly in the central cavity of the ascocarp.

As these developments take place, the central cavity of the ascocarp enlarges further. The peripheral hyphal layer is hardly recognisable; instead, a hyaline layer 2-4 cells in thickness is seen. This is evidently formed by compression of the remnants of the peripheral hyphal mass and may be interpreted as the peridium. The cells of the stroma immediately surrounding this peridium are darkly stained (Fig. 4, m; Plate II, fig. 10).

Usually the initiation of the ascocarps within the stroma is not simultaneous, but successive and irregular. A section of a mature stroma shows the presence of several ascocarps in various stages of development, some being mature, others not (Fig. 5, b; Plate II, fig. 11). The size and the number of ascocarps developing inside the stroma appear to be directly correlated with the size of the stroma.

When an ascus is mature, its wall deliquesces and the ascospores are released. The ascospores from numerous asci now fill the central cavity of the ascocarp (Fig. 6).

The stalk of the stroma is sometimes seen intact until the stroma attains maturity; in most cases, however, it is cast off at an early stage or disappears.

The next phase in the development of the ascocarp is marked by the formation of an opening. The irregularly twisted, thick-walled and more deeply staining hyphae near or around which the young ascocarp was initiated (Fig. 2, d; Plate I, fig. 6) now appear in a less twisted condition (Fig. 6) and are seen in the vicinity of the ascocarp or around it depending on whether the ascocarp was initiated near or within these hyphal masses. Groups of thin filiform hyphae now appear around the ascocarps almost enveloping them (Fig. 6). Sometimes these are seen to be connected to irregular masses of similar hyphae developing elsewhere within the stroma (Fig. 6). Though we do not know the complete sequence of events, we see that ultimately an opening appears for at least some of the ascocarps all of which develop within the stroma and lie buried within it. The development of an opening involves the lysis or breakdown or collapse of cells of the stroma in the region of the opening. How this occurs is not clear. However, it is noteworthy that in some cases a cluster of thin filiform hyphae is seen to emerge through the opening (Fig. 7, a; Plate II, fig. 12). There is a

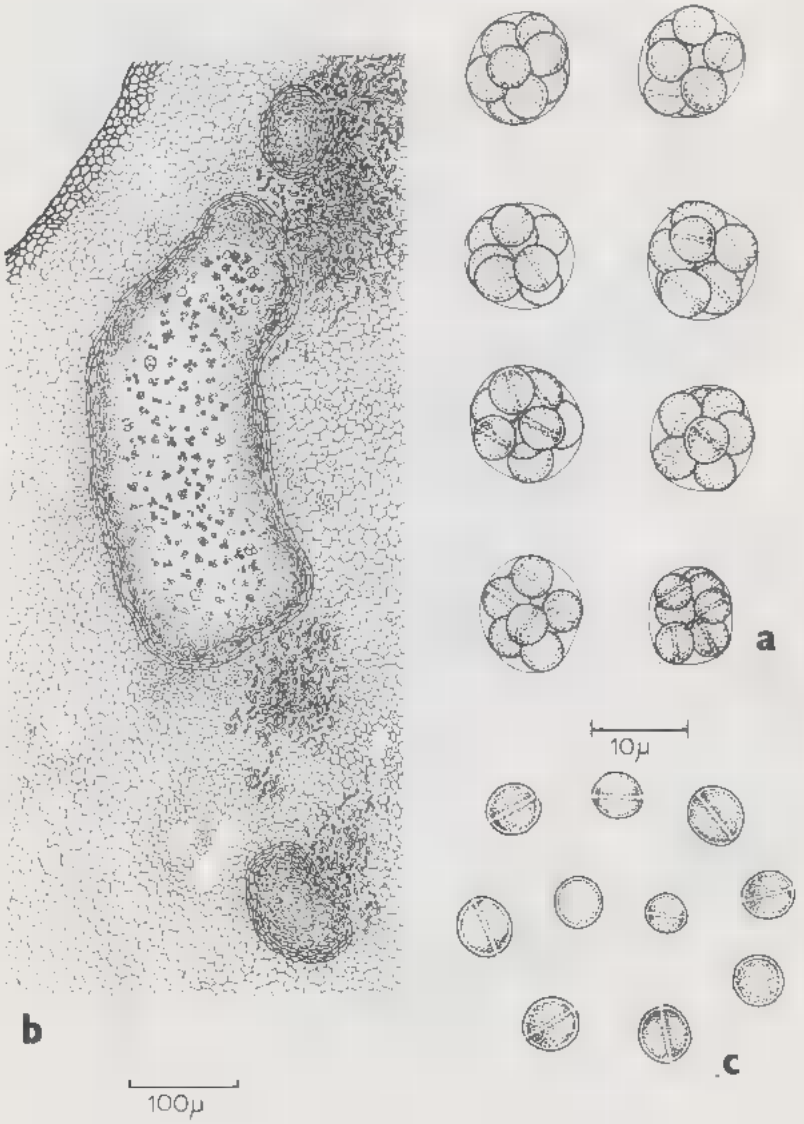

Fig. 5. – *Petromyces alliaceus.* Section of a part of a stroma with three mature ascocarps within.

striking similarity between these hyphae and the filiform hyphae around the ascocarp referred to earlier. We cannot, however, say whether these two are connected in any way. Following the breakdown of the stromal tissue which facilitates the development of an opening, the ascocarp peridium in the region

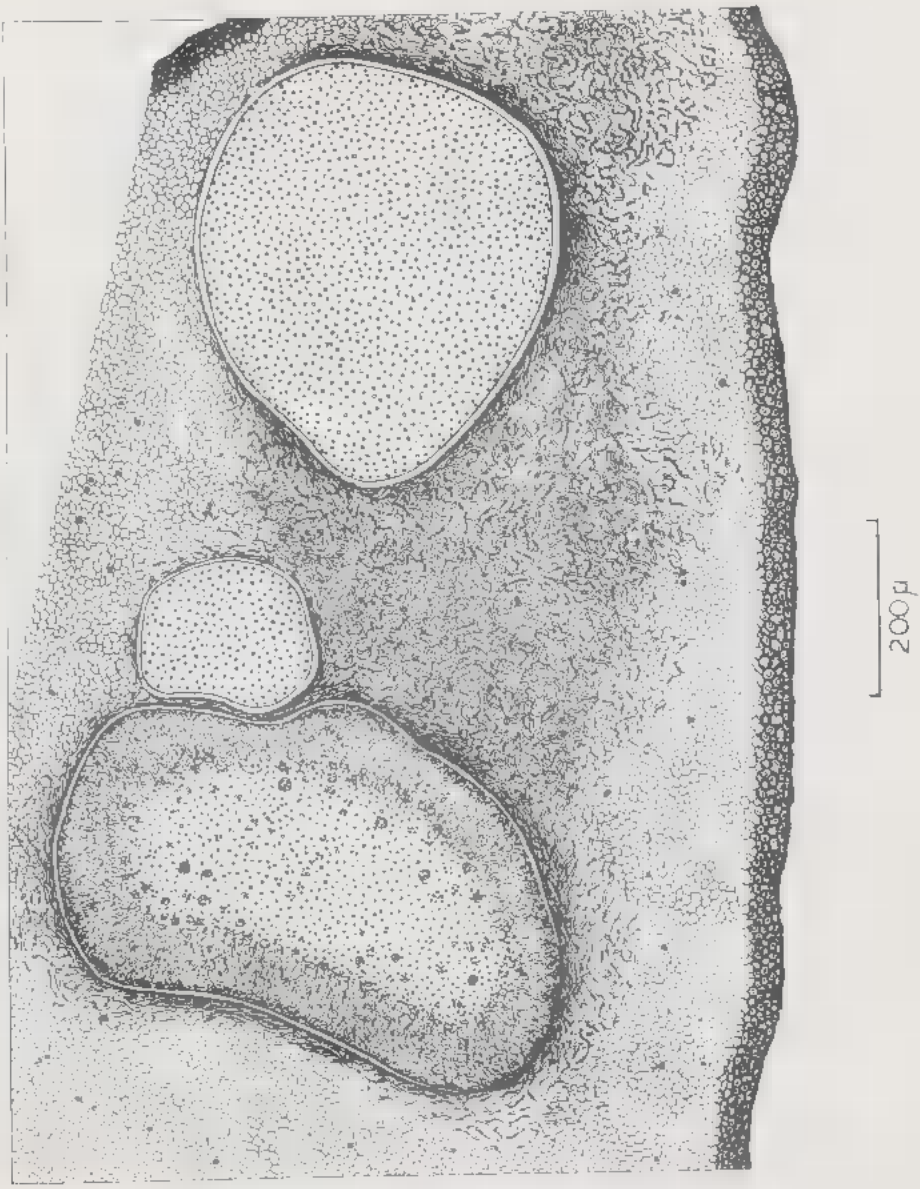

Fig. 6. — *Petromyces alliaceus*. Section of a part of a stroma with 3 mature ascocarps within.

of the opening bulges out into the opening (Fig. 7, b; Plate II, fig. 13), and eventually gets broken off or lysed (Fig. 7, c; Plate II, fig. 14-16), so that the ascospores which now lie free within the ascocarp can be liberated through this opening.

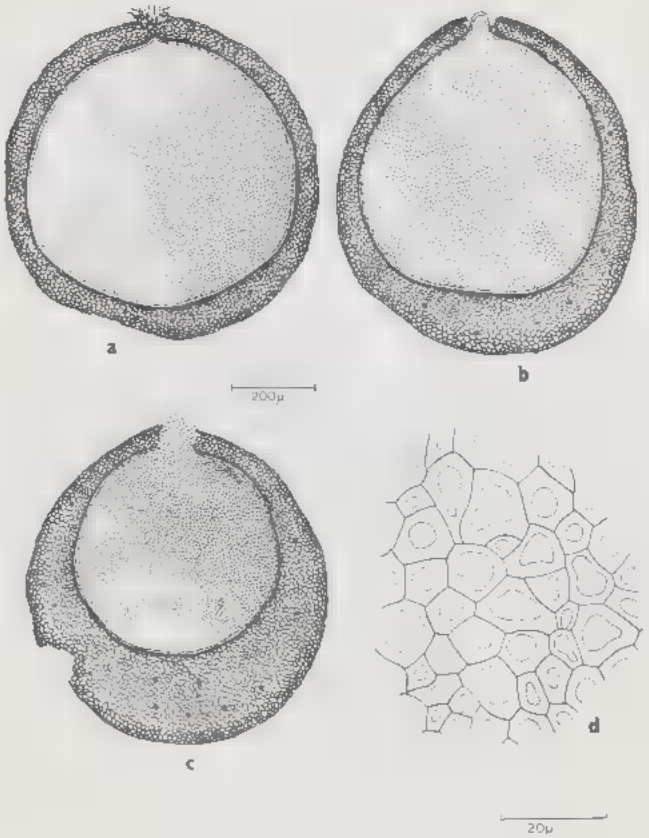

Fig. 7. — *Petromyces alliaceus*. a-c, stroma with a single mature ascocarp showing stages in the development of an opening (section); d, enlarged view of cells of the stroma (section).

The formation of an opening was not observed in all cases; ascocarps which develop deep within the large stromata are possibly without an opening. In such case, as the ascocarp matures, the surrounding pseudoparenchymatous stromal tissue may slowly get disintegrated, followed by the disintegration of the ascocarp peridium; thus, the ascospores get liberated. It must be emphasized, however, only a study of serial sections of ascocarps would throw light on whether all ascocarps have openings or not.

Germination of ascospores

The germination of ascospores is a quick process, taking place within 24 hours of incubation on dialysis tubing placed on malt extract agar medium at a temperature of 23-25°C. Just prior to germination, the ascospore partly

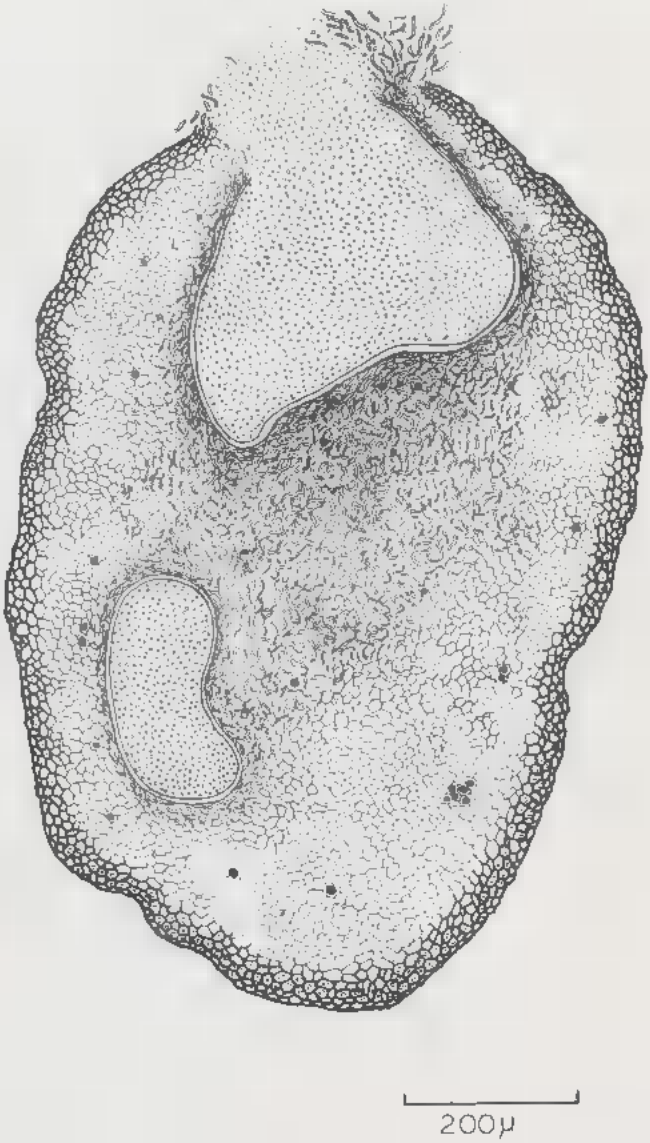

Fig. 8. — *Petromyces alliaceus*. Section of a mature stroma with two ascocarps, one of them with a wide opening through which ascospores are released.

split at one end in the equatorial plane (Fig. 9, a-c) and through the opening so formed, a protrusion emerges from within and assumes a vesicular appearance which is retained in its further development. One or more extensions (germ

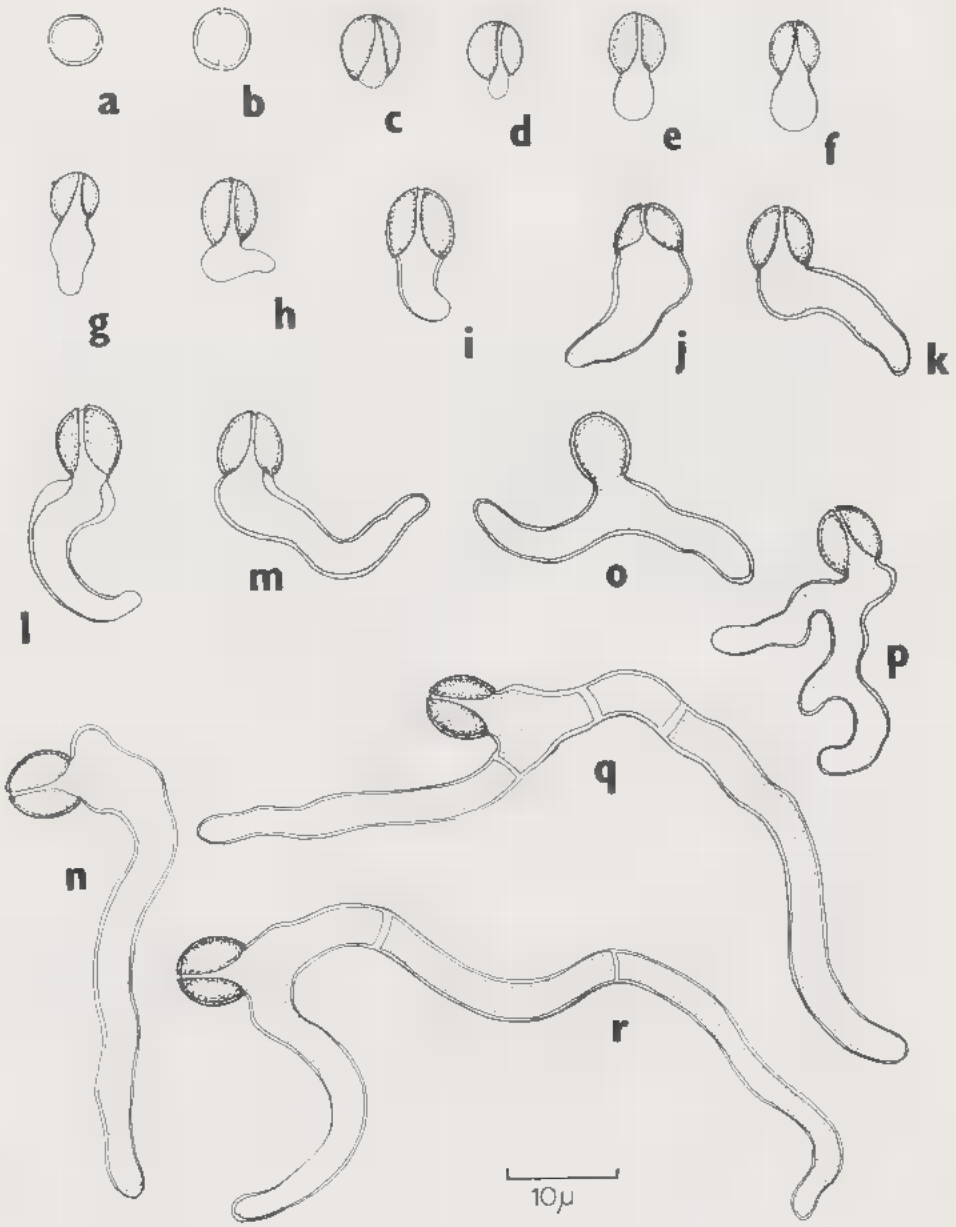

Fig. 9. — *Petromyces alliaceus*. a-r, stages in the germination of ascospores.

tubes) develop from the vesicular part and grow. These branch and become septate to give rise to the vegetative mycelium (Fig. 9, d-r).

DEVELOPMENT OF THE *ASPERGILLUS* STATE

The development of the *Aspergillus* state starts with the production of phialophores as small protuberances from specialized hyphal cells, the foot cells (Fig. 10, a). The foot cells are at first no more than normal hyphal cells, but become thick-walled and assume a variety of shapes as the phialophores arise from them. The phialophores are straight or flexuous and nearly of uniform width throughout.

The young phialophore grows in length, usually perpendicular to the foot cell (Fig. 10, b) and may become flexuous ■ it reaches its full length. The wall of the phialophore is thicker at the base than above. Phialophores are usually non-septate. As the growth of the phialophore ceases, its apical portion becomes swollen to form a somewhat subglobose vesicle (Fig. 10, c).

Several minute protuberances now arise synchronously from the surface of the vesicle, swell and elongate (Fig. 10, d, e). Each elongate protuberance is first cut off from the vesicle by a basal septum (Fig. 10, f). The protuberance then elongates further (Fig. 10, g) and is divided by a septum into an apical cell and a basal cell (Fig. 10, h). The apical cell is the phialide. The basal cell corresponds to the metula. A second phialide now arises as a small bulge immediately below the septum separating the first phialide from the metula (Fig. 10, i, l). This phialide assumes an erect position parallel to the first one. The second phialide is also cut off by a septum from the metula (Fig. 10, m). By ■ repetition of this process, each metula eventually bears several phialides.

The development of the first phialide from each metula appears to be synchronous; similarly the development of the second phialide from each of the metulae also appears to be synchronous (Fig. 10, k).

The conidia develop as follows: the initial of the first conidium appears as a small protuberance at the apex of each phialide (Fig. 10, j, k). The protuberance increases in size as the initial grows into a conidium. With further development of the conidium, the wall of the protuberance which is continuous with that of the phialide wall undergoes circumscissile rupture at the constriction between the protuberance and the body of the phialide (Fig. 10, l, m). The first conidium may be enveloped within the wall of the protuberance. No collarette is visible at the open end of the phialide.

As the first conidium is differentiated, ■ second conidium initial appears below in continuity with the first conidium and pushes it above (Fig. 10, n); a third initial then appears below the second and behaves in the same way. A repetition of these events results in a simple basipetal chain of conidia from the tip of the phialide (Fig. 10, o-x). Each conidium in the chain is separated from adjacent conidia by an isthmus (Fig. 10, z). The isthmi are quite conspicuous.

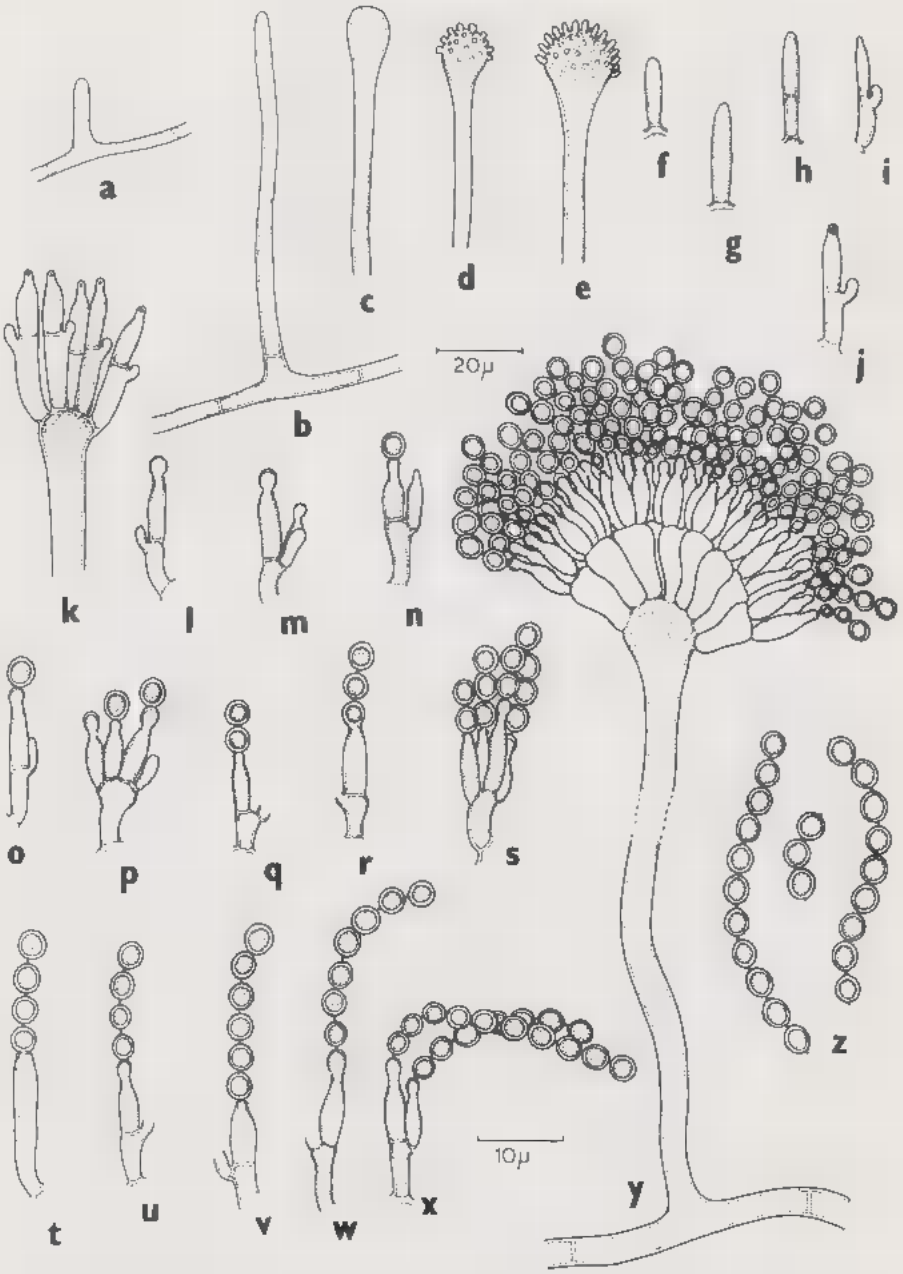

Fig. 10. — *Petromyces alliaceus*. a-k, stages in the development of phialophore, vesicle, metulae and phialides; l-m, stages in the development of the first conidium - note circumsessile rupture of the phialide wall at its tip; n-x, development of the basipetal chains of conidia from the phialide - note isthmi between adjacent conidia; y, mature phialophore with conidial head; z, conidial chains.

TAXONOMY

The genus *Petromyces* Malloch and Cain (MALLOCH and CAIN, 1972) is based on *Petromyces alliaceus* Malloch and Cain.

In *Petromyces alliaceus* one or more ascocarps are differentiated within an ascostroma. Initially, a defoliated-tree-like system of hyphae such as was described for *Warcupiella spinulosa* and *Hamigera avellanea* (SUBRAMANIAN and RAJENDRAN, 1979), develops which, following further branching and close aggregation, becomes a compact ascostroma (Fig. 1, d). Eventually, this becomes pseudoparenchymatous with a distinct rind (Fig. 2, b). Usually, several ascocarps develop within an ascostroma, but sometimes only one ascocarp may develop. Each ascocarp has its own peridium which is pseudoparenchymatous (Fig. 4, n). No detailed information could be obtained in this study about the presence of an ascogonium. The asci are produced from croziers on ascogenous hyphae which develop from the centre outwards. A unique feature of this fungus is the formation of an opening for the ascocarp which involves (?) cracking or (?) lysis of stromal tissue. It is difficult to assess the taxonomic significance of this feature without detailed study of many more taxa of this group.

A comparison of the developmental morphology of the ascocarp in the genus *Eupenicillium* Ludwig (LUDWIG, 1892) with that of *Petromyces* would be pertinent to our theme. The ascocarp of *Eupenicillium* (type species, *E. crustaceum* Ludwig) is a pseudoparenchymatous ascostroma as in the case of *Petromyces*. Within this ascostroma the asci develop and are accomodated in a cavity apparently formed concurrently with the development of the asci. Unlike in *Petromyces*, however, no peridium develops and what is usually considered the peridium is what remains of the original pseudoparenchymatous stroma following the development of the cavity within which the asci develop. In other words, the so-called cleistothecium of *Eupenicillium* is a pseudoparenchymatous ascostroma within which the asci develop in a locule. On the other hand, in *Petromyces* each ascocarp which develops within the pseudoparenchymatous stroma has its own peridium. Moreover, the anamorph of *Eupenicillium crustaceum* is a *Penicillium* and not an *Aspergillus*. Further comparisons with *Eupenicillium* must await more detailed investigation of the developmental morphology of *Eupenicillium crustaceum*.

The genus *Dichlaena* Mont. and Dur. (type species : *D. lentisci* Mont. and Dur.) is another genus apparently very close to *Petromyces* (MALLOCH and CAIN, 1972). Unfortunately, the developmental morphology of this fungus has not been studied at all, and therefore, no critical comparison can be made.

The anamorph of *Petromyces alliaceus* has been studied here in detail and the study indicates that this is correctly placed in the genus *Aspergillus*.

We are grateful to the late Dr. D.I. FENNELL for the culture of *Petromyces alliaceus* on which the present study is based.

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LEGENDS FOR PLATES

Plate I

Petromyces alliaceus. — Fig. 1-3: early stages in the development of the stroma. 1, x 360; 2, x 240; 3, x 230. Fig. 4: section of a young stroma. x 110. Fig. 5: section of a mature stroma showing the development of darkly stained tissue within. x 890. Fig. 6: enlarged view of the darkly stained tissue within the stroma (section) x 960. Fig. 7: development of the ascocarp rudiments within a cavity in the stroma (section) x 210. Fig. 8: enlarged view of a young ascocarp in the stroma showing ascogenous hyphae in the centre surrounded by interwoven narrow hyphae (section) x 360. Fig. 9: a section of a developing ascocarp in a stroma showing enlargement of the central cavity with ascogenous hyphae and croziers within, x 390.

Plate II

Petromyces alliaceus. — Fig. 10: a mature ascocarp with a hyaline peridium within a stroma, x 350. Fig. 11: section of part of stroma showing ascocarps in various stages of development, x 120. Fig. 12 & 13: sections of parts of ascostroma showing the stages in the development of an opening for the ascocarps. 12, x 390; 13, x 530. Fig. 14: stroma with a single mature ascocarp showing the development of an opening, x 80. Fig. 15: section of a mature opening through which ascospores are released, x 60. Fig. 16: a part of same enlarged to show more clearly release of ascospores through the opening, x 80.